CHAPTER 3
MATERIALS AND METHODS

3.1 Study material

3.1.1 Seeds

Seeds of *Brassica juncea* variety RLC-1 were procured from Punjab Agricultural University, Ludhiana, Punjab, India.

3.1.2 24-epibrassinolide (EBR)

EBR was purchased from Sigma Aldrich, St. Louis, USA. A stock solution of $10^5$ nM was prepared by dissolving in EBR in HPLC grade methanol. For experimental purpose, different concentrations of EBR (0, 0.1, 1 and 100 nM) were prepared by serial dilution of the stock.

3.1.3 Imidacloprid (IMI)

IMI was purchased from Sigma Aldrich, St. Louis, USA for preparation of standards for IMI residue analysis. For experimental purpose, IMI 17.8 % S.L. was purchased from K.P.R. Fertilizers limited, Tata Nagar, India. Experimental concentrations of IMI were selected on the basis of IC$_{50}$ values (50% growth inhibition). One concentration lower to IC$_{50}$, IC$_{50}$ and one higher to IC$_{50}$ were selected for experimental work. For laboratory experiments, IMI concentrations selected were 0, 150, 200 and 250 mg IMI L$^{-1}$. For pot experiments, concentrations of IMI were 0, 250, 300 and 350 mg IMI Kg$^{-1}$ soil.

3.2 Combinations of treatments

3.2.1 Laboratory experiments

Combinations of IMI and EBR concentrations used for laboratory experiments are mentioned in table 3.2.1.
Table 3.2.1 Combinations of IMI and EBR selected for laboratory experiments.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IMI (mg L(^{-1}))</th>
<th>EBR (nM L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
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</tr>
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</tr>
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<tr>
<td>7</td>
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<td>1</td>
</tr>
<tr>
<td>16</td>
<td>250</td>
<td>100</td>
</tr>
</tbody>
</table>

3.2.2 Field experiments

Table 3.2.2 gives the detailed combinations of IMI and EBR for field studies.

Table 3.2.2 Combinations of IMI and EBR selected for field experiments.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IMI (mg Kg(^{-1}) soil)</th>
<th>EBR (nM L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<tr>
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<tr>
<td>16</td>
<td>350</td>
<td>100</td>
</tr>
</tbody>
</table>
3.3 Raising of plants

3.3.1 Surface sterilization of seeds

Seeds were surface sterilized by dipping in 0.4% sodium hypochlorite solution for 15 min followed by repeated rinsing with distilled water.

3.3.2 Raising of seedlings in laboratory

Seeds of *B. juncea* (cv. RLC-1) were pre-soaked in EBR solutions (0, 0.1, 1 and 100 nM) for 8 h. Petri-plates were lined with Whatman#1 filter paper containing IMI solutions (0, 150, 200 and 250 mg L\(^{-1}\)). The EBR pre-soaked seeds were rinsed with distilled water before sowing in Petri-plates containing IMI solutions. The Petri-plates were kept in seed germinator for seed germination at 25±0.5°C (photoperiod = 16 h, light intensity = 175 µmol m\(^{-2}\) s\(^{-1}\)). The seedlings were harvested for further analysis after 10 days of seed sowing.

3.3.3 Raising of plants in field

Seeds of *B. juncea* var. RLC1 were given pre-sowing treatment for 8h with EBR solutions (0, 0.1, 1 and 100 nM). Pots were filled (6 pots for each combination) with 8 Kg soil containing clay, sand and manure in the ratio of 2:1:1. Pots filled with soil were given pre-sowing treatment of pesticide concentrations as mentioned in table 3.2.2. After rinsing with distilled water, seeds soaked with EBR were sown in IMI treated soils. Plants were irrigated with ground water as per need. In order to avoid the edge effect, positions of the pots were interchanged after every two weeks. Leaves of the plants were harvested for further analysis after 30, 60, 65 and 90 days of seed sowing. Green pods were harvested 80 days after seed sowing.

3.4 Analysis of growth parameters

Seedling and shoot length were measured using “cm” scale. Seedling and shoot biomass were determined by weighing after drying the samples at 70 °C for 48 h. Number of leaves per plant were counted. All the parameters were studied using 10 samples.
Materials and Methods

3.5 Pigments and gaseous exchange parameters

3.5.1 Chlorophyll content

Chlorophyll contents were estimated according to Arnon (1949).

Procedure

One g fresh leaves were ground well in a pestle and mortar with 4 ml of 80% acetone and centrifuged at 1500×g for 20 min (4 °C). The supernatant was collected and utilized for the quantification of chlorophyll and carotenoid contents. The absorbance values of the supernatant for chlorophyll content were taken at 645 and 663 nm.

Calculations

Total chlorophyll, chlorophyll-a, and chlorophyll-b contents were calculated using below mentioned equations and were expressed as mg g⁻¹ fr. wt.

\[
Total\ Chlorophyl = \{20.2 (Abs_{645}) + 8.02 (Abs_{663})\} \times \left(\frac{v}{1000 \times w}\right)
\]

\[
Chlorophyl\ a = \{12.7 (Abs_{663}) - 2.69 (Abs_{645})\} \times \left(\frac{v}{1000 \times w}\right)
\]

\[
Chlorophyl\ b = \{22.9 (Abs_{645}) - 4.68 (Abs_{663})\} \times \left(\frac{v}{1000 \times w}\right)
\]

Where \(v\) = plant extract volume (ml)

\(w\) = plant sample weight (g)

3.5.2 Carotenoid content

Carotenoid content was estimated according to Maclachlan and Zalik (1963).

Procedure

One g fresh leaves were homogenized with 4 ml of 80% acetone followed by centrifugation at 1500×g for 20 min (4 °C). The supernatant was used to estimate...
carotenoid content by taking absorbance at 480 and 510 nm using UV-visible spectrophotometer.

Calculations

Calculations of carotenoid content (mg g\(^{-1}\) fr. wt.) were made as follow

\[
Carot.\ content = [7.6 (Abs_{480}) - 1.49 (Abs_{510})] \times \left( \frac{\nu}{1000 \times w} \right)
\]

Where \(\nu = \) volume of plant sample extract (ml)

\(w = \) fresh weight of plant sample taken (g)

3.5.3 Anthocyanin content

Anthocyanin content was assessed according to Mancinelli (1984).

Procedure

Fresh leaves (1 g) were uniformly homogenized in 3 ml of extraction mixture (2.37 ml methanol, 0.6 ml water, 0.03 ml HCl). The crushed material was then centrifuged at 1500\(\times\)g for 20 min (4 °C). The supernatant absorbance was taken at 530 nm and 657 nm using UV-visible spectrophotometer.

Calculations

Calculations were done using following equations and anthocyanin content was expressed as \(\mu g\ \text{g}^{-1}\ \text{fr. wt.}

\[
A = Ab_{530} - (0.25 \times Ab_{657})
\]

\[
\text{Anthocyanin content} = \frac{(A \times \text{Mol. Wt.} \times DF \times 1000)}{\varepsilon}
\]

Where \(\text{Mol. Wt.} = \) molecular weight (cyanidin-3-glicoside, 449.2)

\(DF = \) dilution factor

\(\varepsilon = \) molar absorptivity (cyanidin-3-glicoside, 26900)

3.5.4 Xanthophyll content

Xanthophyll content was estimated by the AOAC method described by Lawrence (1990).
**Materials and Methods**

**Procedure**

50 mg of dried leaf powder was taken in a 100 ml flask. To this, 30 ml of extractant containing 10 ml hexane, 7 ml acetone, 6 ml absolute alcohol, 7 ml toluene was poured and then shaken well for 10-15 min. The flask was refluxed in water bath at 56°C for 20 min after adding 2 ml of 40% methanolic KOH. The flask was then reserved in dark for 1 h and 30 ml of hexane was then pipetted in the flask. Flask was then shaken for 1 minute and the final volume 100 ml was made up with 10% solution of sodium sulphate. The flask was again shaken for 1 minute. The flask was kept in dark for 1 h. 50 ml volumetric flask was used to collect the upper phase and the volume was made up using hexane, mixed well and absorbance was measured at 474 nm using UV-visible spectrophotometer.

**Calculations**

Calculations were done as follow and xanthophyll content was expressed as mg g⁻¹ dr. wt.  

\[ \text{Xanthophyll content} = \frac{(\text{Abs}_{474} \times D)}{(W \times 236)} \]

Where \( W \) = sample taken (g)  
\( D \) = final dilution  
236 = specific-absorptivity (trans-lutein in g/l)

**3.5.5 Gaseous exchange parameters**

The following gaseous exchange parameters were measured after 30, 60 and 90 days of seed sowing using LI-COR LI-6400XT portable open photosynthesis system.  

- Photosynthetic rate (\( P_n \))  
- Stomatal conductance (\( G_s \))  
- Inter-cellular \( CO_2 \) (\( C_i \))  
- Transpiration rate (\( E_t \))
Materials and Methods

Procedure

In open system IRGA, air is allowed to enter into analysis and reference lines from same source at constant \( \text{CO}_2 \) level. Measurements of gaseous exchange parameters (\( P_n, C_i, E_t \) and \( G_s \)) are based upon the differences in the levels of \( \text{CO}_2 \) and \( \text{H}_2\text{O} \) in air flowing into the reference chamber in comparison to air coming out of sample chamber.

The measurements of all gaseous exchange parameters were taken in morning hours from 10:00 to 11:00 am. Instrument operating conditions were as follow:

Air temperature = 25 °C

Photon flux density = 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)

Air relative humidity = 80-90%

\( \text{CO}_2 \) concentration = 400 \( \mu \text{mol mol}^{-1} \)

3.6 Antioxidative defense system

3.6.1 Estimation of the contents of oxidative stress markers

3.6.1.1 Superoxide anion (\( \text{O}^\cdot_2 \))

Superoxide anion content was estimated according to Wu et al. (2010).

Procedure

One g of plant tissue was homogenized in 6 ml of phosphate buffer (65 mM, pH = 7.8) containing 1% of polyvinylpyrrolidone. Homogenate was centrifuged at 5000xg for 15 min at 4 °C. To 0.5 ml of supernatant, 0.5 ml of phosphate buffer (65 mM, pH = 7.8) and 0.1 ml of hydroxylamine hydrochloride (10 mM) were added. Mixture was incubated at 25 °C for 30 min. After incubation, 1 ml of 3-aminobenzenesulphonic acid (58 mM) and 1 ml of 1-naphthylamine (7 mM) were added to mixture followed by incubation at 25 °C for 20 min. The absorbance was taken at 530 nm. To calculate superoxide content, standard curve of sodium nitrite was used and content was expressed as \( \mu \text{mole g}^{-1} \) fr. wt.

3.6.1.2 Hydrogen peroxide (\( \text{H}_2\text{O}_2 \))

\( \text{H}_2\text{O}_2 \) was evaluated using method given by Patterson et al. (1984).
**Materials and Methods**

**Procedure**

Plant tissue (0.5 g) was crushed in 1 ml of acetone followed by centrifugation at 5000×g for 15 min at 4 °C. To supernatant, 20 µl of 20% titanium chloride in concentrated HCl was added. Then 200 µl of ammonia solution (17 M) was added, followed by repeated washing of the precipitates with acetone. Washed precipitates were dissolved in 1.5 ml of H₂SO₄ (2 N). Absorbance was read at 410 nm. Content of hydrogen peroxide was calculated from standard curve of H₂O₂ and was expressed as µmole g⁻¹ fr. wt.

**3.6.1.3 Malondialdehyde (MDA)**

Method given by Heath and Packer (1968) was used to estimate MDA content.

**Procedure**

Fresh plant tissue (1 g) was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) followed by centrifugation at 10000×g for 15 min at 4 °C. To 1 ml of supernatant, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added. Mixture was incubated at 95 °C for 30 min followed by cooling in ice and centrifugation at 10000×g for 15 min at 4 °C. The absorbance of supernatant was taken at 532 and 600 nm. To calculate MDA content, absorbance value at 532 was subtracted from absorbance value at 600 in order to correct unspecific turbidity and extinction coefficient of 155 mM⁻¹ cm⁻¹ was used.

**3.6.2 Estimation of activities of antioxidative enzymes**

3 mL of 100 mM potassium phosphate buffer (PPB) with a pH of 7.0 was used for homogenization of 1 g of fresh plant tissue. The homogenate was then centrifuged at 12000g for 20 min at 4 °C. Storage of supernatant (sample) was done at -20°C and used for further determination of antioxidative enzymes activities including CAT, APOX, POD, GPOX, GR, DHAR and GST.

**3.6.2.1 Catalase (EC 1.11.1.6)**

CAT activity was estimated according to Aebi (1984) with slight modifications.
Materials and Methods

Procedure

In cuvette, the reaction mixture consisted of 1500 µl of PPB (pH = 7.0, 50 mM), 930 µl of hydrogen peroxide (15 mM) and 70 µl of sample. Absorbance was taken at 240 nm.

Calculations

Activity of CAT was calculated using following equations

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

Specific activity (µmole min}^{-1} \text{ mg}^{-1} \text{ protein}) = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}

Where, Extinction co-efficient is 43.6 M}^{-1} \text{ cm}^{-1}

3.6.2.2 Ascorbate peroxidase (EC 1.11.1.11)

Ascorbate peroxidase (APOX) activity was evaluated according to Nakano and Asada (1981).

Procedure

70 µl sample with 2130 µl PPB (50 mM, pH 7), 200 µl ascorbate (0.5 mM) and 100 µl H₂O₂ (1.0 mM) were used to prepare reaction mixture. Absorbance was taken at 290 nm.

Calculations

APOX activity was calculated using below mentioned equations

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

Specific activity (µmole min}^{-1} \text{ mg}^{-1} \text{ protein}) = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}

Where, Extinction co-efficient is 2.8 mM}^{-1} \text{ cm}^{-1}
3.6.2.3 Glutathione peroxidase (EC 1.11.1.9)

Glutathione peroxidase (GPOX) activity was analyzed according to Flohé and Günzler, 1984.

**Procedure**

Reaction mixture containing 1180 µl PPB (50 mM, pH 7.0), 0.5 mM of 250 µl ethylene diamine tetraacetate (EDTA), 250 µl reduced glutathione (1.0 mM), 250 µl sodium azide (1.0 mM), 250 µl nicotinamide adenine dinucleotide phosphate (NADPH) (0.15 mM), 250 µl H2O2 (0.15 mM), 2.5 units of GR and 70 µl sample was used. Absorbance was read at 340 nm.

**Calculations**

Activity of GPOX was calculated as follow

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

\[
\text{Specific activity (µmole min}^{-1} \text{ mg}^{-1} \text{ protein)} = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}
\]

Where, Extinction co-efficient is \(6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\)

3.6.2.4 Guaiacol peroxidase (EC 1.11.1.7)

Activity of Guaiacol peroxidase (POD) was determined using Putter (1974).

**Procedure**

Absorbance was recorded at 436 nm by preparing a reaction mixture containing 70 µl sample with 2350 µl PPB (50 mM, pH 7.0), 50 µl guaiacol solution (20 mM) and 30 µl H2O2 (12 mM).

**Calculations**

Following equations were used to calculate POD activity
Materials and Methods

Unit activity (Unit min⁻¹ g⁻¹ fr. wt.)

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

Specific activity (\(\mu\text{mole min}^{-1} \text{ mg}^{-1} \text{ protein}\)) = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}

Where, Extinction co-efficient is 25.5 mM⁻¹ cm⁻¹

3.6.2.5 Dehydroascorbate reductase (EC 1.8.5.1)

Activity of DHAR was measured by following the method described by Dalton et al. (1986) with modifications

Procedure

Reaction mixture comprised of 1330 µl of PPB (pH = 7.0), 300 µl of dehydroascorbate (0.2 mM), 300 µl of EDTA (0.1 mM), 500 µl of reduced glutathione (2.5 mM) and 70 µl of plant sample. The absorbance of reaction mixture was read at 265 nm.

Calculations

Activity of DHAR was determined using following equations

Unit activity (Unit min⁻¹ g⁻¹ fr. wt.)

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

Specific activity (\(\mu\text{mole min}^{-1} \text{ mg}^{-1} \text{ protein}\)) = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}

Where, Extinction co-efficient is 14 mM⁻¹ cm⁻¹
3.6.2.6 Glutathione reductase (EC 1.6.4.2)

Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik (1975).

**Procedure**

Reaction mixture contained 1530 µl PPB (50 mM, pH 7.0), 300 µl each of EDTA (3.0 mM), NADPH (0.1 mM), oxidized glutathione (1.0 M), and 70 µl sample. Reading was taken at 340 nm.

**Calculations**

Below mentioned equations were used to estimate GR activity

\[
\text{Unit activity} \ (\text{Unit min}^{-1} \ g^{-1} \ \text{fr. wt.}) = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

\[
\text{Specific activity} \ (\mu\text{mole min}^{-1} \ mg^{-1} \ \text{protein}) = \frac{\text{Unit activity} \ (\text{Unit min}^{-1} \ g^{-1} \ \text{fr. wt.})}{\text{Protein content (mg g}^{-1} \ \text{fr. wt.)}}
\]

Where, Extinction co-efficient is 6.22 mM\(^{-1}\) cm\(^{-1}\)

3.6.2.7 Glutathione-S-transferase (EC 2.5.1.18)

Glutathione-S-transferase (GST) activity was quantified based on Habig and Jacoby (1981).

**Procedure**

For final reaction mixture 1930 µl PPB (50 mM, pH 7.5), 250 µl each reduced glutathione (10 mM), 1-chloro-2,4-dinitrobenzene (10 mM), was added to 70 µl sample. Absorbance was measured at 340 nm.
Calculations

Activity of GST was determined as follow

\[
\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)} = \frac{\text{Change in abs min}^{-1} \times \text{Total volume (ml)}}{\text{Extinction coeff.} \times \text{Volume of sample (ml)} \times \text{Wt. of tissue (g)}}
\]

\[
\text{Specific activity (µmole min}^{-1} \text{ mg}^{-1} \text{ protein)} = \frac{\text{Unit activity (Unit min}^{-1} \text{ g}^{-1} \text{ fr. wt.)}}{\text{Protein content (mg g}^{-1} \text{ fr. wt.)}}
\]

Where, Extinction co-efficient is 9.6 mM\(^{-1}\) cm\(^{-1}\)

3.6.2.8 Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) activity was estimated according to Kono (1978) with minor modifications.

Procedure

One g of plant tissue was homogenized in 3 ml of sodium carbonate buffer followed by centrifugation at 12000×g at 4 °C for 20 min. Supernatant was used as sample for further analysis.

Reaction mixture consists of 1630 µl of sodium carbonate buffer (pH = 10.2), 500 µl of nitroblue tetrazolium (24 µM), 100 µl of EDTA (0.1 mM), 100 µl of hydroxylamine hydrochloride (1 mM), 100 µl of Triton-X-100 (0.03%) and 70 µl of sample. Absorbance was taken at 560 nm.

Calculations

SOD activity was calculated from the percent inhibition of nitroblue tetrazolium reduction

\[
x = \frac{\text{Change in abs min}^{-1} \text{ (blank)} - \text{Change in abs min}^{-1} \text{ (sample)}}{\text{Change in abs min}^{-1} \text{ (blank)}} \times 100
\]

Where, \(x\) = percent inhibition caused by 70 µl of sample.

Hence 50% of inhibition is caused by \(\frac{50 \times 70}{x} = y \ µl \ of \ sample\)

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3.6.3 Estimation of the contents of non-enzymatic antioxidants

Fresh plant tissue (1g) was homogenized in 3 ml of tris buffer (50 mM, pH 10.0) containing 1 mM EDTA. The homogenate was then subjected to centrifugation at 5000×g for 15 min and the supernatant from plant extract was used for antioxidant content analysis (ascorbate, glutathione and tocopherol).

3.6.3.1 Ascorbate

Ascorbic acid content was determined following the method given by Roe and Kuether (1943).

Procedure

To the mixture containing 4 ml double distilled water, 0.5 ml plant extract, and 0.5 ml 50% of trichloroacetate (TCA) 100 mg of activated charcoal was added. After mixing well it was filtered with Whatman filter paper#1. 0.4 ml of 2,4-dinitrophenylhydrazine (DNPH) reagent was added to the 1 ml of filtrate obtained, and the mixture was incubated at 37 °C for 3 hours followed by cooling in ice bath. 1.6 ml of cold H₂SO₄ (65%) was then added and reserved at room temperature for 30 min. Absorbance was taken at 520 nm.

Calculations

Following equation was used to estimate ascorbate content (mg g⁻¹ fr. wt.)

\[
\text{Ascorbate content} = \frac{\text{Abs. of sample} \times \text{Conc. of standard} \times \text{Total vol.}}{\text{Abs. of standard} \times \text{Vol. of sample}}
\]

3.6.3.2 Glutathione

The glutathione content was determined according to the method given by Sedlak and Lindsay (1968).

Procedure

To the 100 μl of plant extract, 1 ml of tris buffer, 50 μl of Ellman’s reagent and 4 ml of absolute methanol were added, and kept at room temperature for 15 minutes for incubation and then subjected to centrifugation at 12000×g for 15 min. The absorbance of the supernatant obtained was noted at 412 nm.
Materials and Methods

Calculations

GSH content was estimated according to below mentioned equation (mg g\(^{-1}\) fr. wt.)

\[
Glutathione\ content = \frac{Abs.\ of\ sample \times Conc.\ of\ standard \times Total\ vol.}{Abs.\ of\ standard \times Vol.\ of\ sample}
\]

3.6.3.3 Tocopherol

Tocopherol content was determined following the method given by Martinek (1964).

Procedure

Equal volumes of plant extract, absolute ethanol and double distilled water i.e. 0.5 ml each were mixed. To this mixture 0.5 ml of xylene was added and shaken vigorously, followed by centrifugation at 12000×g for ten minutes. After this 0.5 ml of upper layer obtained was mixed with 0.5 mL of 2,4,6-tripyridyl-s-triazine (TPTZ) reagent and the absorbance was read at 600 nm.

Calculations

Following equation was used to estimate tocopherol content (mg g\(^{-1}\) fr. wt.)

\[
Tocopherol\ content = \frac{Abs.\ of\ sample \times Conc.\ of\ standard \times Total\ vol.}{Abs.\ of\ standard \times Vol.\ of\ sample}
\]

3.6.3.4 Polyphenols

Polyphenol profiling was done according to Sharma et al. (2016a)

Procedure

For polyphenols estimation, 1 g of leaf sample was homogenized in 5 ml of 80% methanol and 15 minutes of centrifugation at 8000×g. For polyphenol analysis 10 µl of extract was injected in Shimadzu UHPLC system (Nexera) with SPD-M20A photodiode array detector (wavelength 280 nm) and C\(_{18}\) analytical column (flow rate 1 ml min\(^{-1}\)). Mobile phase was composed of 0.01% acetic acid (phase A) and 100% methanol (phase B). HPLC grade solvents were used.
3.6.3.5 Total phenols

Total phenolic content was estimated according to method described by Singleton and Rossi (1965).

Procedure

1 g plant material was crushed in 5 mL of 60% ethanol, and then incubated at 60°C for 30 minutes. 1.25 mL of Folin–Ciocalteu reagent and 1 ml of 7.5% Na₂CO₃ was added to and mixed with 250 μl incubated extract. After 2 hours of incubation at room temperature, the absorbance of the mixture was recorded at 765nm.

Calculations

Total phenol content was estimated using standard curve of gallic acid solution (standard) and was expressed in mg g⁻¹ dr. wt.

3.7 Estimation of proteins, amino acids and organic acids

3.7.1 Protein estimation

Protein estimation was done according to Lowry et al. (1951)

Reagent preparation

Reagent A consists of sodium carbonate (2%) in sodium hydroxide (0.1 N) Reagent B consists of copper sulphate (0.5%) in potassium sodium tartrate (1%). Reagent C consists of 100 ml of reagent A and 2 ml of reagent B.

Procedure

One g of fresh plant tissue was crushed in 3 ml potassium phosphate buffer (50 mM, pH = 7.0) and centrifuged at 10000×g for 20 min at 4 °C. Supernatant (0.1 ml) was added to test tube and 1 ml of distilled water was added. Now 5 ml of reagent C was poured to test tube and incubated at room temperature for 10 min. After this, 0.5 ml of Folin–Ciocalteu reagent was added to the test tube followed by incubation at room temperature in dark for 30 min. Absorbance was read at 660 nm. To determine the protein content, standard curve was used and the content was expressed as mg g⁻¹ fr. wt.
3.7.2 Amino acid profiling

Sample preparation

Amino acid profiling was done according to Iriti et al. (2005) with minor modifications. One g of fresh plat tissue was crushed in 5 ml methanol (80%) followed by centrifugation at 10000×g for 20 min at 4 °C. To 1 ml of supernatant, 1 ml of sulphosalicylic acid (6%) was added and mixture was centrifuged at 10000×g for 20 min at 4 °C. To 1 ml of sample after centrifugation, 200 µl of HCl (0.1 N) was added followed by sample filtration using 0.22 µm syringe filters.

Analysis using amino acid analyzer

Amino acid profiling was done using amino acid analyzer (Shimadzu, Nexera X2). To analyze the samples, mobile phase- A contained phosphate buffer (pH 5.6), mobile phase-B contained acetonitrile, methanol and ultrapure water (in the proportion 9:8:3), mobile phase-R0 contained 80% methanol and mobile phase-R3 contained 20% acetonitrile. To wash analytical column, 0.1% formic acid dissolved in 50% methanol was used. Derivatization of samples was done using mercaptopropionic acid buffer, O-phthalaldehyde and 9-fluorenymethyl chloroformatein. Sample volume used for injection was 1 µl, analytical column used was silica-bonded amino-acid column C18.

3.7.3 Estimation of organic acids using GC-MS

Sample preparation

Method given by Chen et al. (2001) was altered to estimate organic acids using GC-MS. Extraction of the organic acids was done by adding 0.5 ml each of 0.5 N HCL and of methanol to 50 mg of dried seedling powder followed by 3 h shaking and then centrifugation at 10000×g for 10 min. 300 µl of methanol and 100 µl of 50% H2SO4 were added to the supernatant obtained, followed by overnight incubation in water bath at 60 °C. The mixture was cooled down to 25 °C after that 800 µl of chloroform and 400 µl of distilled water were added to it followed by vortexing for 1 min. For estimation of organic acids, lower chloroform layer was used.
Analysis using GC-MS

For evaluation of organic acid content, 2 µl of chloroform layer was injected into GC-MS system (Shimadzu GC-MS-QP2010 Plus). Helium was used as a carrier gas, initial column temperature was 50 °C (held for 1 min) which was raised at 25 °C/min to 125 °C followed by further increase at 10 °C/min to 300 °C and held for 15 min. Injection temperature was 250 °C and mode of injection was split. Flow of gas in the column was 1.7 ml min⁻¹, and analytical column used was DB-5ms. Ion source temperature was set at 200 °C and interface temperature set was 280 °C. Quantification of organic acids was done using standard curve.

3.8 Analysis of elemental composition and phytochemical analysis

3.8.1 Elemental analysis using SEM-EDX

One g of oven dried leaves (100 °C for 48 h) were grounded to obtain fine powder before coating with silver. Elemental analysis was done using scanning electron microscope-energy dispersive X-ray spectrometer (SEM-EDX, model- Zeiss, Supra-55).

3.8.2 Phytochemical analysis using GC-MS

3.8.2.1 Seedlings

Sample preparation

0.5 g of fresh seedlings were homogenized with 0.5 ml of extractant (1% acetic acid in acetonitrile) and 0.2 g of MgSO₄ and 0.1 g of NaOAc was added (To remove excessive water from sample). The mixture was then centrifuged at 5000×g for 15 min. To the upper centrifuged layer (0.25 ml), 0.035 g of MgSO₄ and 0.015 g of primary secondary amine (to remove excessive fatty acids and sugars) were added. The mixture was again centrifuged at 13000 RPM for 15 min. Upper phase was used for GC-MS analysis.

Analysis using GC-MS

8 µl of sample was injected into Shimadzu GC-MS-QP2010 Plus for phytochemical analysis. **Conditions:** Carrier gas: helium, initial column oven
temperature was 50 °C, then raised to 125 °C at 25 °C min\(^{-1}\) and finally increased to 300 °C at 10 °C min\(^{-1}\) and held for 15 min. Injection temperature: 250 °C, injection mode: splitless and column flow: 1.70 ml min\(^{-1}\). DB-5ms analytical column was used. Ion source temperature: 200 °C and interface temperature: 280 °C. Compounds were identified using NIST08s and Wiley7 library.

3.8.2.2 Leaves

Sample preparation

For phytochemical profiling, separate extraction of 1 g of fresh leaves was done with 50 ml of ethanol. The extracts were then dried out at 50 °C using rotary evaporator followed by further reconstruction of the residues to 2 ml with methanol.

Analysis by GC-MS

Leaf extracts of *B. juncea* L. were analyzed using Shimadzu GCMS-QP2010 Plus. Helium gas was used as the carrier gas, the injection temperature was set at 280 °C, initial column temperature was 70 °C, held for 6 minutes, and then increased to 250 °C, finally increased to 300 °C at the rate of 10 °C min\(^{-1}\), being held for 10 minutes.. Injection mode set was split with 1 minute sampling time, linear flow control mode, 110.8 KPa pressure, 38.9 ml min\(^{-1}\) total flow and 1.71 ml min\(^{-1}\) column flow, 47.2 cm sec\(^{-1}\) linear velocity, analytical column used was DB-5ms with 30 m length and 0.25 mm id. Ion source temperature was 250 °C and interface temperature was 290 °C.

Identification of the phytochemicals

National Institute of Standard and Technology (NIST08s) and Wiley7 library were used to compare the detected phytochemicals using mass spectra.

3.9 Gene expression and pesticide residue analysis

3.9.1 Gene expression analysis

100 mg of whole seedlings was used for total RNA extraction through Trizol method based on manufacturer’s protocol (Invitrogen). Total RNA of 1 μg was used for reverse transcription through RNA to cDNA kit (Invitrogen) containing MuLV as reverse transcriptase, dNTP’s mix, random octamers and oligo (dT), based on
manufacturer’s instructions. Gene specific primers (Table 3.9.1) were designed using mRNA sequence of particular gene obtained from Genbank and EMBL database and due to high expression stability in vegetative stage of plants *actin* gene was used as an internal control. Quantitative real time PCR (qRT-PCR) was performed using the StepOne™ real time detection system (Applied Biosystems) and Power SYBR® Green PCR Master Mix (ThermoFisher SCIENTIFIC). At the end of the each PCR cycle, a melting curve was generated, with the software provided along with the PCR system which verified that a single product was amplified. The mRNA quantification was based on the method of Livak and Schmittgen (2001). For obtaining ΔCt values the threshold value (Ct) of the internal control (*actin*) was subtracting from that of the gene of interest. ΔΔCt values were obtained by subtracting the Ct values of the untreated control sample from the ΔCt value. The fold changes in expression level comparative to the untreated samples were expressed as $2^{-\Delta\Delta Ct}$.

### Table 3.9.1 Primers used for quantitative real time polymerase chain reaction (qRT-PCR).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Gene</th>
<th>Forward primer sequence</th>
</tr>
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<tbody>
<tr>
<td>actin</td>
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<td>GST-5</td>
<td>5’AGTGGCTGCAAAAGCTTGT 3’</td>
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<td>GST-6</td>
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<td>POD</td>
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<td></td>
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<td>5’AACCCTCCATGAAGGACT 3’</td>
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<tr>
<td>GR</td>
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<td>P450</td>
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<tr>
<td></td>
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<td></td>
<td>5’CACAACGAGTTGCAGATG 3’</td>
</tr>
<tr>
<td>RBO</td>
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<td>CS</td>
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<tr>
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<td>5’TGTGGAAGAACAACCA 3’</td>
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### Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Gene</th>
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<td><strong>SUCLG1</strong></td>
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<tr>
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<td>TCCGCGTTGATTTTATCTC 3'</td>
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<tr>
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<td><strong>PAL</strong></td>
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<td><strong>GST-4</strong></td>
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<td>5'TGGTCAGTGGTCAAGCCATA 3'</td>
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<td>AGCGAAACATGAGCTCTC 3'</td>
</tr>
</tbody>
</table>

*SOD = superoxide dismutase, CAT = catalase, GR = glutathione reductase, RBO = respiratory burst oxidase, RUBISCO = Ribulose-1,5-bisphosphate carboxylase/oxygenase, NADH = NADH-ubiquinone oxidoreductase, CXE = carboxylesterase, GSH-S = glutathione synthase, GSH-T = glutathione transporter-1, GST = glutathione-S-transferase, POD = guaiacol peroxidase, P450 = cytochrome P450 monooxygenase, CS = citrate synthase, SUCLG1 = succinyll Co-A ligase, SDH = succinate dehydrogenase, FH = fumarate hydratase, MS = malate synthase, CHLASE = chlorophyllase, PSY = phytoene synthase, CHS = chalcone synthase, and PAL = phenylalanine ammonialyase.*
3.9.2 IMI residue analysis

Sample preparation for IMI residue analysis

For IMI residue analysis, fresh plant material was taken and processed based on AOAC official method 2007.01 (2007). This extraction process includes crushing of 1 g of fresh plant sample with 1 ml of 1% acetic acid in acetonitrile. 0.5 g of anhydrous magnesium sulfate (MgSO$_4$) and sodium acetate in the ratio of 4:1 w/w per g of sample were added to the extract, and shaken vigorously, followed by centrifugation for 1 min at 1500xg. After that the dispersive solid phase extraction was done. For this, anhydrous MgSO$_4$ and primary secondary amine sorbent (In the ratio of 3:1 w/w, for 1 ml of acetonitrile extract, 200 mg of mixture is added) was taken and added to the upper layer of centrifuged sample and shaken vigorously and again centrifuged to obtain supernatant.

Analysis by GC-MS

To determine the pesticide residue, the centrifuged sample (supernatant) was now transferred to separate vials and injected into the Shimadzu GC-MS-QP2010 Plus. **Conditions:** GC: The Carrier gas was helium; the column furnace temperature was set at 50 °C, increased to 125 °C at 25 °C min$^{-1}$, again amplified to 300 °C at 10 °C min$^{-1}$ and held for 15 min. Following settings were made: injection temperature 250 °C, injection mode split, sampling time 1 min, flow control mode linear, pressure 100.1 KPa, total flow 30.0 ml min$^{-1}$, column flow 1.70 ml min$^{-1}$, linear velocity 47.2 cm sec$^{-1}$, purge flow 5.0 ml min$^{-1}$, sample injection volume 8 μl, analytical column used DB-5ms having length 30 m and 0.25 mm id. **MS:** Ion source temperature was set at 200 °C, interface temperature was 280 °C, solvent cut time was 3 min and detector gain mode was relative.

3.10 Statistical analysis

3.10.1 Standard deviation

Square root of the variance is known as standard deviation and was calculated using following formula.
$S.D. = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}$

Where, $x_i =$ observation

$\bar{x} =$ mean

$n =$ number of observations

### 3.10.2 Analysis of variance

Two-way analysis of variance (ANOVA) was applied to the data using self-coded softwares in MS-Excel 2010. Null hypothesis tested was that at least two means are significantly different from each other at particular p value. To calculate honestly significant difference (HSD) values between two means ($p<0.05$), Tukey’s multiple comparison test was applied.

### 3.10.3 Multiple linear regression analysis (MLR)

In order to find the relationship between dependent and independent variables, multiple linear regression was applied as mentioned in the equation given below:

$$y = a + b_1 x_1 + b_2 x_2$$

Where $y =$ dependent variable

$a =$ y-intercept

$b_1 =$ partial regression coefficient for $x_1$ on $y$ eliminating effect of $x_2$

$b_2 =$ partial regression coefficient for $x_2$ on $y$ eliminating effect of $x_1$

$x_1 =$ independent variable (IMI)

$x_2 =$ independent variable (EBR)

(Self-coded softwares in MS-Excel 2010).

#### 3.10.3.1 Beta-regression coefficients

Unitless $\beta$-regression coefficients ($\beta_{\text{IMI}}$ and $\beta_{\text{EBR}}$) explained the relative effects of independent variables (IMI and EBR) on dependent variable (Self-coded softwares in MS-Excel 2010).
3.10.4 Artificial neural network analysis (ANN)

ANN model is comprised of an input layer, one or more than one hidden layers and an output layer (Fig. 3.10.1). The input layer represents the independent variables (IMI, EBR and DAS). Dependent variables are taken as targets (Statistica-12 software).

In this model, weights from input to hidden layer approach the neurons and each neuron gets an input bias resulting in a weighted sum which is activated by a function ($\tanh$). After activation, weighted sum combines with a hidden bias to give output. ANN model was fitted to obtain correlation between simulated and experimental values. Number of neurons was automatically selected by the software at maximum variance explained. The other specifications were, 1 hidden layer, 1000 iterations and $\tanh$ transfer function from both input to hidden, and hidden to output layers.

Neuron number in the hidden layer was automatically decided by software based upon number of independent variables (Statistica-12). Generally number of neurons in ANN model are 70 to 90% of input layer (Boger and Guterman, 1997). The other options were multilayer perceptron (MLP), one each input, hidden, and target layer, 1000 iterations, 3-4 neurons, $\tanh$ transfer function from both input to hidden, and hidden to output layers.
Fig. 3.10.1 Artificial neural networks (ANN) model with IMI, EBR and DAS as inputs. (IMI= applied imidacloprid, EBR= applied 24-epibrassinolide, DAS=days after sowing, n=neuron).